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Organ Pretreatment with Cytotoxic Endothelial Localising Peptides To Ameliorate Microvascular Thrombosis & Perfusion Deficits In Ex- vivo Renal Haemo-reperfusion Models

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All authors have no conflicts of interest to disclose.

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Abbreviations:

CIT	cold ischaemia time
D	diameter
DCD	donation after cardiac death
DGF	delayed graft function
ECD	extended criteria donor
FCD	functional capillary density
HMP	hypothermic machine perfusion
IR	ischaemia reperfusion
mM	millimolar
NHRP	normothermic ex-vivo renal haemo-reperfusion
OPS	orthogonal polarization spectral imaging
PFI	perfusion flow index
PI	perfusion index
RBC	red blood cell
rsMD	rapid sampling microdialysis
SCS	static cold storage
TLN	thrombalexin
UW	University of Wisconsin solution
WIT	warm ischaemia time
µm	micrometre

Abstract:

Background: Hypothermic machine organ perfusion (HMP) offers opportunity to manipulate grafts with pharmacological agents prior to transplantation. Pretreating organs with novel cytotoxic anti-coagulant peptides that localise to endothelial cell membranes could ameliorate microvascular thrombotic sequelae posttransplantation. We describe experiments testing Thrombalexin (TLN), a novel cell binding thrombin-inhibitor, using porcine and unused human kidneys in a series of ex-vivo normothermic haemo-reperfusion models.

Methods: 38 porcine kidneys were utilised. Control kidneys underwent pretreatment via HMP with either unmodified perfusion solution (n=15) or solution with Inactive-TLN (absent anticoagulant effect, n=4). Test kidneys were perfused with TLN treated solution (n=19). All kidneys then underwent haemo-reperfusion. Two unused human kidneys underwent a similar protocol.

Results: HMP pretreatment facilitated delivery and tethering of TLN in the organ microvasculature. Haemo-reperfusion challenge demonstrated improved perfusion in TLN-treated kidneys compared to controls: 26.4% superior flow (30.6 vs.23.1 ml/min/100g,p=0.019) and 28.9% higher perfusion flow indices (0.43 vs.0.32 ml/min/100g/mmHg,p=0.049). Orthogonal polarisation spectral imaging demonstrated superior microvascular capillary perfusion in TLN-treated organs vs. controls (9.1 vs. 2.8pl/s/mm²,p=0.021). Rapid-sampling microdialysis for cortical [lactate] as a marker of tissue ischaemia/metabolism detected lower levels in TLN-treated kidneys. Perfusate analysis demonstrated reduced fibrin generation in TLN-treated kidneys correlating with perfusion data.

Conclusion: Our data suggest HMP graft pretreatment with cytotoxic anticoagulants is feasible and ameliorates perfusion deficits seen in ex-vivo haemo-reperfusion models. There is potential for further development and application of this translational strategy to deliver locally-active anti-coagulants directly within grafts and decrease microvascular thrombotic sequelae, while avoiding systemic anticoagulation and its associated risks.

ACCEPTED

Introduction:

'Organ painting' is a process of organ pretreatment prior to transplantation with therapeutic agents that are able to localise to the membranes of endothelial cells of the organ vasculature, 'painting' the organ from the 'inside'.

Such compounds can be termed 'cytotoxic' [1] and such targeting has recently been applied in organ transplantation, with the design Mirococept (or APT070) a complement inhibitor molecule designed with cell binding functionality[2,3].

Mirococept has been evaluated in animal models of limb-ischaemia, renal and cardiac transplantation to ameliorate both ischaemia reperfusion (IR) injury and rejection[4], and is currently undergoing MRC funded Phase 2 trials (EMPIRIKAL) in the UK in kidney transplantation.

Serine plasma proteases of the coagulation cascade have been recently highlighted as potentially important and previously under-appreciated contributors in the pathogenesis of IR injury[5,6]. These developments indicate a complex interplay between endogenous coagulation and inflammatory processes, characterised by endothelial damage and generation and deposition of intravascular fibrin, propagation of which causes occlusion of the microvasculature, microcirculatory failure and the 'no re-flow' phenomenon[7,8]. In addition, overt renal allograft thrombosis occurs in 0.1-8.2% of renal allografts[9] and is a detrimental complication that is variably implicated in 7-44% of all cases of early graft loss in adults[10,11].

With the increasing use of ECD and DCD organs to address the growing need of patients on transplant waiting lists, the impact of IR injury is seen by the higher rates of DGF in these more susceptible organs and this impacts on the acceptability of their use by transplant surgeons [12].

However use of the DCD/ECD cohort could expand the donor pool by 40%[13]. Interventions that can improve organ viability, and improve function in the peri-operative period during which IR injury is most dominant are potential strategies to better utilise these donor organs[14]

Heparin has been used extensively in prevention of thrombosis in transplanted organs in particular kidney and pancreas allografts[15–17] although the risk of bleeding is significant, and prevention of thrombosis not guaranteed[15]. Therefore, an ideal approach would be a cytotoxic that localises within the vasculature of the allograft, exerting an anticoagulant (and thus anti-inflammatory) effect locally at the site of the IR response rather than systemically. Manipulating an organ in such a targeted way might negate the need for systemic anti-coagulation and be potentially more effective.

To this aim we have utilized a membrane-associated anticoagulant derived from a minimized form of hirudin, (a known thrombin inhibitor, and naturally occurring peptide in the salivary glands of *Hirudo medicinalis* leeches). The principle underlying this synthetic cell-tethered inhibitor (Thrombalexin, TLN)[18] is similar to the complement inhibitor Mirococept[4]. TLN binds the cell phospholipid bilayer through a bis-myristoyl hydrophobic structure and a lysine-rich sequence[19], and like hirudin TLN interacts with thrombin at the active site and at the fibrinogen-binding exosite I[20].

Here in we describe a series of experiments, building on our experience[21] with hypothermic machine perfusion (HMP) to evaluate the potential of ex-vivo organ pretreatment with TLN in ameliorating microvascular coagulation and both macro and micro vascular perfusion deficits in a series of normothermic ex-vivo renal haemo-reperfusion (NHRP) circuits. These experiments

utilised both porcine kidneys, and unused human kidney grafts allocated by NHS Blood and Transplant for research.

Methods:

Experiments

Four experiments were conducted and compared TLN to controls. Experiments 1-3 involved porcine kidneys. Experiment 2 incorporated additional assessment of porcine kidneys during NHRP, specifically orthogonal polarisation spectral (OPS) imaging, and rapid sampling microdialysis (rsMD). Experiment 4 involved use of human kidney grafts allocated for use in this research.

Details of the experimental groups and the protocols used are summarised in Figure 1. All experiments were conducted in laboratories of the Department of Surgery at Imperial College London in collaboration with MRC Centre for Transplantation at King's College London.

Organ Retrieval and Hypothermic Machine Perfusion (HMP)

Kidneys were retrieved from landrace pigs terminated at a local abattoir by stunning and exsanguination. This model of sacrifice was akin to substantial head injury and also DCD as animals were only accessed once cardiac death had occurred. There was a target warm ischaemia of approximately 15 minutes until the institution of cold flush. Access was achieved through a midline laparotomy incision, kidneys were retrieved via en-block dissection, flushed with preservation solution (500-1000ml at 100cmH₂O) and packed on ice for transport to the laboratory. Paired kidneys were matched between treatment and control groups. Static cold

storage time prior to HMP including transportation was on average 5 hours. All kidneys were perfused initially on a Waters Medical Systems RM3 device for 4 hours of HMP stabilization with UW solution according to clinical protocols[22].

Human allografts

Approval was granted for research use of human kidney grafts initially retrieved but subsequently not used for transplantation by a National Research Ethics Committee (IRAS code:84202) and NHS Blood and Transplant (the national regulator of organ transplantation in the United Kingdom). One pair of organs were used in this study as part of a direct preclinical model in experiment 4, and underwent identical perfusion protocols as porcine kidneys.

Cytotopic Anti-coagulant

TLN is a conjugate peptide of the direct thrombin inhibitor [D-Phe-L-Pro-L-Arg-L-Pro-Gly-Gly-Gly-L-Asp-Gly-L-Asp-L-Phe-L-Glu-L-Glu-L-Ile-L-Pro-L-Glu-L-Glu-L-Tyr-L-Leu-Gly-Gly-L-Cys-amide] and the cytotopic-tail APT3146 [(N-alpha,-N-epsilon-*Bis*-myristoyl-Lys-Ser-Ser-Lys-Seryl-Pro-Lys-Lys-Asp-Asp-Lys-Lys-Pro-Gly-Asp-(S-2-pyridyldithio) Cys)][19]. The peptide had a mass of 4649 Daltons as determined by time-of-flight mass spectrometry and was prepared by reaction of the 2 components in aqueous solution followed by further purification[18]. A fluorescently labelled-version, lacking anticoagulant activity (FAM-PTL), was used to study localisation of TLN within the kidney. The component peptides were prepared using solid phase synthesis by Cambridge Research Biochemicals, Billingham, UK and the conjugate also synthesized at scale by Almac Sciences, Craigavon, UK.

TLN Pretreatment

After HMP stabilization, kidneys underwent pretreatment with TLN. This consisted of 3 distinct 30 minutes hypothermic perfusion episodes (diagrams summarising this protocol and experimental groups can be seen in Figure 1). A translational HMP setting was chosen to facilitate delivery of TLN into grafts mimicking what would potentially occur in clinical practice.

- 1) Washout-1: 30 minutes HMP with fresh perfusate to limit the amount of remaining donor blood in the organ and perfusate from the initial 4 hours of HMP;
- 2) Treatment: perfusion with perfusate containing anticoagulant active-TLN (Protein Test Groups 1-4; PTG1-4); or as controls, either un-modified perfusate (Control Groups 1, 2 and 4; CG1, CG2, CG4) or perfusate with anticoagulant inert 'tail-only' proteins (Tail Control Group 4, TCG4); and
- 3) Washout-2: 30 minutes HMP with fresh perfusate to facilitate removal of non-adherent TLN from the organ vasculature.

To provide for an ideal control environment 1 kidney from each pair underwent 'treatment' with TLN while the other served as 'untreated' control.

Normothermic Haemo-reperfusion

Kidneys underwent 6 hours of normothermic haemo-reperfusion (NHRP) using minimally anticoagulated whole blood (WB) perfusate (10,000U/L Heparin); 600mls, 3:1 ratio WB:0.9% saline. RM3 units were setup to facilitate NHRP (see Figure 1). Human grafts were also perfused

with porcine whole blood to establish the effect of TLN treatment in a haemo-reperfusion model. This phase of reperfusion mimics the injury suffered in vivo.

OPS Imaging

OPS (Cytometrics, Inc.; Philadelphia, USA) was used to assess the microcirculation[23]. OPS is based on cross-polarisation of reflected green (548nm) light by oxy- and deoxy-hemoglobin in RBCs, enabling blood vessels to be visualized and assessed for perfusion metrics including capillary diameter (D), functional capillary density (FCD), RBC velocity (RBCv), with a perfusion index (PI) calculated [24]. See Supplementary Information, SDC, <http://links.lww.com/TP/B337>.

rsMD

A novel in-house rsMD system previously described [25] for in-vivo assessment of tissue biochemistry and metabolism was used to evaluate renal tissue biochemistry during HMP stabilization and NHRP. Lactate levels were analysed in real-time (every 60s). Through microdialysis probes (MAB11.35.4, Microbiotech, Sweden) inserted superficially into the parenchyma of the lateral cortex, lactate levels were analysed in real-time (every 60s).

Clotting Assays

Perfusate samples during pretreatment were assessed via a simple calcium chloride/human plasma clotting assay. Blood perfusate samples during NHRP were collected and analysed for levels of fibrin degradation products (INNOVANCE® D-Dimer Siemens Healthcare Diagnostics), fibrinogen and Prothrombin Factor 1+2 (Quadrach Diagnostics Ltd, UK) using commercially available kits.

Histology

Wedge biopsies were taken for histopathological analysis after 6 hours of NHRP for routine staining. Frozen sections were used for immuno-histochemistry for fibrin(ogen) (No. 350, American Diagnostica Inc). Samples were evaluated using a scale of 0-4 for endothelial detachment, presence of tubular debris/tubular intratubular cell detachment, tubular dilatation, and peritubular vessel congestion according to the degree of changes seen in each field of view at 10x magnification.

TLN Adherence

Two porcine kidneys and 1 human kidney were treated with a TLN-tail derivatised at the N-terminus with a FAM fluorescent derivative (FAM-PTL) to histologically demonstrate tethering and adherence to the kidney microvasculature.

Statistics

Values are represented as mean and standard deviation. Continuous variables were plotted as the mean area under the curve and evaluated using the paired students T-test or Wilcoxon signed rank test, and repeated measures analysed using ANOVA for repeated measures with Bonferroni correction, as required. Data and statistical analysis was performed using Microsoft® Excel Software (Reading, UK). P value of ≤ 0.05 was considered significant.

Results:

Kidney Characteristics

Thirty-eight porcine kidneys and 2 human kidney grafts were utilized and Table 1 summarises porcine kidney characteristics in the experimental groups.

Hypothermic Machine Perfusion Stabilization

Perfusion characteristics and weight gain in kidneys during initial HMP stabilization were similar between the Protein Treated Groups (PTG1,PTG2,PTG3) and the Control Groups (CG1, CG2, TCG3), and are summarised in Table 2.

TLN Delivery and Adherence during pretreatment phase

Perfusate samples taken during the 'treatment' phase of organ pretreatment from TLN treated kidneys (PTG1, PTG2 and PTG3 Groups) were assessed using a simple clotting assay that indirectly measured the concentration of TLN in the perfusion fluid. These data are shown in Figure 2. A higher clotting ratio indicates a greater concentration of TLN present in the sample. In all experiments, the concentration of TLN fell during the treatment phase, consistent with uptake in the kidney. During the washout-2 phase, clotting ratios increased, consistent with washout of non-adherent TLN.

Adherence of TLN

Porcine and human grafts treated with FAM-PTL histologically confirmed tethering and adherence to kidney microvasculature after pretreatment, with continued adherence after 6 hours of NHRP (Figure 3).

NHRP

TLN treated kidney groups demonstrated superior blood flow and PFI compared to the Control Group kidneys (Figure 4). Renal blood flow rates were on average 26.4%, 31.7%, and 45% higher, and PFIs 28.9%, 25.1%, and 42.5% greater in protein treated PTG1, PTG2 and PTG3 kidneys compared to controls.

Orthogonal Polarisation Spectral Imaging

In Experiment 2 OPS data was recorded and analysed for 4 pairs of kidneys after 6 hours NHRP to assess the microdynamics of the kidney microvasculature. FCD was similar between treated and non treated kidneys however TLN treated kidneys had 44% larger diameter capillaries, 50% faster RBCv, 3.5 times greater CBFs and PIs compared to the nontreated control kidneys (Figure 5).

Rapid Sampling Microdialysis

Data of cortical micro-dialysis levels were recorded for 4 pairs of kidneys during Experiment 2 during the HMP stabilization phase, and NHRP. During HMP cortical microdialysis lactate concentrations were similar between the nontreated control and TLN-treated kidneys (CG2: 0.128 ± 0.006 mM/min vs. PTG2: 0.137 ± 0.04 mM/min, $p=0.783$). In NHRP cortical lactate levels were higher in non-treated vs. TLN-treated kidneys (CG2: 3.28 ± 1.04 mM/min vs. PTG2: 1.67 ± 0.28 mM/min, $p=0.072$), with the difference approaching significance (Figure 6).

NHRP Coagulation Analysis

Coagulation data is summarised in Table 2. Higher levels of D-Dimers (average AUC) were observed over the 6 hours of perfusion in the Experiment 1 control group, CG1 (14.5 ± 7.9 mg/L) vs. the TLN-treated kidneys, PTG1 (9.5 ± 5.1 mg/L, $p=0.051$) with this difference at verge of significance. No other differences were observed in coagulation parameters between TLN-treated and nontreated kidneys in Experiments 2 and 3.

Histology

Light microscopy histological assessment of biopsy samples between Control (CG1-2, TCG3) and TLN-treatment (PTG1-3) Groups showed no significant differences in endothelial detachment, tubular debris/epithelial detachment, tubular Dilatation, and peritubular congestion, all p-values >0.5. Immuno-histochemistry for fibrin(ogen) demonstrated a generalised fibrin(ogen) deposition in interstitial tissues and peritubular capillaries, with no difference between treated and nontreated kidney grafts.

Pre-clinical Experimentation with Human Kidney Grafts

A pair of DCD kidney grafts were used in a preclinical model in Experiment 4. CIT was 78 hours (4680 minutes) until start of the experiment. Table 3 summarizes kidney characteristics, perfusion dynamics, perfusate clotting results and microdialysis cortical lactate levels in these kidneys. During HMP stabilization perfusion characteristics were superior in the CG4 control kidney vs. the PTG4 kidney.

During NHRP despite similar systolic perfusion pressures (CG4: 73.2mmHg vs. PTG4: 72.9mmHg) TLN treated kidneys demonstrated better perfusion with 17% higher renal blood flow and perfusion indices. Renal blood flow in the PTG4 group was 70.8 ml/min/100g vs. CG4: 60.7 ml/min/100g, and PFI: in PTG4: 0.97 ml/min/100g/mmHg vs. CG4: 0.83 ml/min/100g/mmHg. Micro-dialysis lactate concentrations were lower in the control kidney during HMP prior to treatment but were subsequently 36% lower in the treated PTG4 kidney during NRHP. D-Dimer and fibrinogen levels were 2.5-3x higher over the 6 hours of perfusion in the control CG4 group. Percent weight changes, and reperfusion injury demonstrated by light microscopy were similar.

Discussion:

The increasing use of ECD and DCD organs to expand donor pools means any approach that could ameliorate micro-vascular dysfunction and improve organ perfusion at the reperfusion stage is in high clinical demand. TLN is a novel membrane localising 'cytotoxic' anti-coagulant construct that has proven efficacy in improving graft function in murine kidney transplantation models[26]. This is the first study of the pretreatment of large animal organs with this cytotoxic anti-coagulant agent using HMP as a delivery vehicle, and further assessing the benefits of this pre-treatment using a NHRP challenge. Using a series of translational porcine ex-vivo renal perfusion and haemo-reperfusion models we demonstrated HMP can be used to deliver TLN into the vasculature of a kidney graft, and facilitates cytotoxic tethering and adherence; improving macro and micro-vascular organ tissue perfusion, with evidence of its anticoagulant activity during NHRP challenge.

HMP successfully delivered TLN into the kidney vasculature, with tethering and adherence. The decrease in clotting time ratios using the simple plasma-CaCl based clotting assay in samples from Experiment 1 taken as the treatment phase progressed demonstrates removal of TLN from the perfusate and sequestration onto the vasculature, while at the same time demonstrating functionality of the HLL anti-coagulant moieties. Similar trends were seen Experiment 2 and 3 demonstrating removal of TLN from the perfusate. Cytotoxic protein adherence in these experiments was confirmed using the FAM-PTL analogue. This qualitatively demonstrated the presence of the TLN after treatment, with continued adherence to the kidney microvasculature and ultra-structure after 6 hours of NHRP challenge when using the perfusion protocols in these experiments. Furthermore allied work in rodent models the effective residence time of TLN is approximately 24 hours (unpublished data). The translational approach using hypothermic

delivery of TLN into grafts mimics the potential strategy that could be employed clinically in organ preservation where HMP is increasingly becoming common-place versus simple cold storage. With the recent advent of normothermic perfusion preservation, as this new technology develops into wider use in clinical practice both in the UK and international settings there is increasing potential for the feasible delivery of localising agents in a more physiological and normothermic environment, and this is an area of ongoing research at our institution.

Isolated organ reperfusion circuits using autologous blood have developed as ex-vivo methods to experimentally assess organ viability in large animal models without the need for live auto-transplantation[27]. The normothermic haemo-reperfusion phase of the protocol used minimally heparinised autologous whole porcine blood. The aim of the NHRP protocol was to induce a haemodynamic procoagulant insult to the kidney to allow study of the impact of the presence of the TLN tethered to the kidney endothelium. Induction of clotting was demonstrated indirectly by the detection of increasing D-Dimers levels as reperfusion progressed (data not shown), and was especially marked in the control kidneys, indicating progressive fibrinolysis. This was associated with histological evidence of tubular and endothelial damage, and a generalised and diffuse pattern of fibrin(ogen) deposition, which is characteristic of IR injury[28].

Immediately after transplantation there is systemic activation of the coagulation system[29]. The subsequent complex interface between coagulation and inflammation mediates reperfusion damage to the endothelium, and subsequent microcirculatory failure. This provokes further ischaemia and tissue injury manifested as DGF, with a higher risk of immunologic rejection[30]. The impact of thrombin inhibition has been recently demonstrated in a porcine kidney autotransplantation model of DGF, as a direct thrombin inhibitor (melagatran) improved autograft function and survival when added either to UW during static cold-storage or

administered systemically[31] . Because thrombin has both procoagulant and inflammatory effects, thrombin inhibition can ameliorate both these sequelae.

Our experiments suggest that TLN anchored to the endothelium can inhibit the activation of clotting. This resulted in superior macro-vascular and micro-vascular perfusion metrics (renal blood flow, PFI) in TLN-treated kidneys compared to the nontreated and Tail-only protein treated controls. Macro-vascular PFI is a useful calculation as it incorporates both renal flow rates and systolic pressure but corrects for differences in kidney mass. In these experiments observed renal blood flow rates and PFIs were between 25-45% superior in the TLN-treated kidneys vs. controls. Relative flow rates during ex-vivo NHRP can be considered indicators of relative organ viability[32].

Ameliorating the microcirculatory perfusion dysfunction that is seen in renal IR injury in both experimental and clinical settings has been shown to be important and beneficial in improving initial graft function after transplantation, and limiting DGF[33–35].

OPS of the microcirculation demonstrated better perfusion in TLN-treated kidneys during the NHRP challenge. The improved calculated microvascular PI (pl/s/mm²) in these kidneys stemmed from larger diameter cortical capillaries, and faster blood flow through these vessels.

The presence of larger calibre vessels can be due to relative vessel dilation[36] secondary to potentially less thrombin action at the endothelial cell surface, and less generation of local vasoconstrictors[37] (eg endothelin) or alternatively more release of nitric oxide (NO). Alternatively the larger capillaries could be due to less fibrin formation and aggregation of cellular blood components at the endothelial surfaces, rendering better transit through vessels[24]. The larger observed diameter in these vessels may relate to a relative inhibition of

dynamic leucocyte aggregation at the endothelial due to less thrombin mediated inflammatory activation, and diminished fibrin generation. Fibrin itself is known to be a potent proinflammatory mediator[38].

These changes correlated with the trend for reduced D-Dimer levels during NHRP challenge in TLN-treated kidneys. Although our data approached significance in only 1 experiment this may have been due to confounding effects of the sacrifice used in this uncontrolled DCD model (where a cytokine surge from stunning may render blood more procoagulant), and where D-Dimers levels are only an indirect measure of thrombin activity.

Faster RBC velocity could be attributed to the relatively lower intravascular clotting activity and less obstruction to flow in capillaries. Maintaining equal systolic perfusion pressures between control and TLN-treated kidneys during NHRP dictates that any observed differences in renal perfusion blood flow dynamics (blood flow rates, PFI) are due to inherent differences in the kidney vasculature and effect of the presence of the cytotopic anti-coagulant proteins. The similar changes in weight in kidneys during HMP and NHRP further indicates that perfusion differences between treated and nontreated grafts are not due to tissue oedema limiting blood flow.

These changes in microcirculatory parameters also associated with lower levels of tissue lactate detected via rsMD in TLN-treated kidneys, most likely reflecting better overall tissue perfusion, both in relative oxygen delivery, and potentially washout of toxic metabolites[39,40]. The higher variability seen in the control group reflects the variation in graft damage sustained during sacrifice and retrieval, and is an inherent component of an uncontrolled DCD model and may be a reason for the lack of statistical significance.

Furthermore rsMD analysis was available only for 4 pairs of kidneys due to technical difficulties with the rsMD system itself. Localization of the rsMD probes to the cortex was done visually without image guided confirmation introducing the possibility of placement of part of the probe in an alternate region of the kidney parenchyma with differing metabolic profiles to the cortex[25].

These data complement those obtained in other model systems illustrating the benefits of localised anticoagulation. Inhibition of thrombin in hearts of transgenic mice[41] was associated with complete resistance to acute humoral rejection[42] in a highly stringent xenogeneic model. Similarly, building on the work of Ding et al[43], Crikis et al reported improved graft survival of transgenic hearts from mice expressing human thrombomodulin localised to endothelium[44]. In the same study these transgenic mice demonstrated a reduced inflammatory response in stasis-induced thrombosis and a mouse-to-rat xenograft model, with reduced HMGB1 levels in LPS induced endotoxaemia. These reports demonstrate the translational potential of targeting anticoagulants to graft endothelial cells and to inhibit coagulation and inflammation.

Use of appropriate preclinical models is a prerequisite to the development of protocols for clinical introduction. The data obtained from TLN preconditioned human kidneys correlated with our findings in the porcine models. These experiments utilized porcine blood during the NHRP challenge phase, and as evidenced by the progressive increase in fibrin degradation products during NHRP in the control kidneys this combination generated ideal conditions under which the impact of TLN could be studied.

The cytotoxic approach suggested here using HMP to deliver TLN is novel and theoretically ideal. An allograft could undergo HMP at the recipient transplant centre, where TLN would be

added to the perfusate, to facilitate tethering and pretreat the endothelium. The allograft would ultimately be transplanted with the thrombin inhibiting anti-coagulants localised to the allograft vasculature, limiting the need for systemic anti-coagulation (with associated bleeding risks) and be potentially more effective. The thrombin inhibiting effects of the TLN would ideally reduce both the procoagulant and proinflammatory actions of thrombin - ameliorating IR responses, and improve postoperative allograft perfusion and function.

Cytotopic painting of the endothelium with TLN can be considered a potential example of 'immunocamouflage' where by the cell membrane surface is modified by nonimmunologic molecules creating a barrier[45]. Although the definitive mechanism of the effect of TLN in this model has not been demonstrated here, it is most likely through inhibition of thrombin by the hirulog sequence contained within; this would be consistent with all in-vitro and ex-vivo testing performed on TLN so far. However, we cannot completely rule out a direct effect due to interaction with the glycocalyx and other membrane associated proteins.

The other caveat to be applied to interpreting this work is that the histological outcomes did not reveal remarkable differences in the treated grafts, and this is likely due to the experimental time frame employed. Despite use of isolated organ reperfusion circuits as well established initial alternatives to in-vivo experimentation the relatively short duration of ex-vivo reperfusion is not a sufficient timescale for development of histological changes that can be seen in-vivo during transplantation[46] with the predominant effects of reperfusion in this model on cellular and molecular processes within the graft. Extending experimentation and reperfusion time would allow further development of histological sequale and the use of a large animal porcine auto-transplant model would also allow for functional graft assessment.

Conclusion:

For the first time the pretreatment of kidney grafts with cytotopic therapeutics using HMP, in ex-vivo porcine and preclinical human kidney perfusion models has been successfully demonstrated, and the presence of these agents improves organ perfusion. There is a high potential for application of this cytotopic technique to clinical practice, not solely for kidney transplantation, but for also for other organs, for example pretreating marginal pancreatic allografts which are very susceptible to reperfusion injury and subsequent allograft thrombosis.

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Figure/Table Legends:

Figure 1 Schematic summary of experimental protocols for TLN testing in porcine and human renal grafts illustrating the 3 distinct phases: HMP stabilization, TLN treatment and Normothermic haemo-reperfusion. Experiments 1 and 2 were consecutive experiments with similar perfusion protocols and the addition of rsMD and cytoscan assessment of grafts during reperfusion. A photograph of the normothermic haemoreperfusion reperfusion circuits using modified Waters Medical RM3 perfusion device is shown. SCS: static cold storage, HMP: hypothermic machine perfusion, CG: Control Group, PTG: Protein Test Group, UW: University of Wisconsin Solution.

Figure 2. Perfusate Clotting Analysis. This method was used as an indirect analysis of the amount of TLN present in the perfusion fluid. Clotting times are expressed as multiple of the clotting time for a control sample of unaltered perfusate (represented by the blue line). The larger the value the greater the concentration of TLN present. Experiment 1: ANOVA $p=0.001$, 0 mins (2.19 ± 0.17) vs. 15 mins (1.89 ± 0.12) $p=0.001$, 30mins (1.92 ± 0.11) vs. start washout (1.15 ± 0.04) $p<0.001$, start vs. end of washout (1.26 ± 0.1) NS, 30mins vs. end of washout $p<0.001$. Experiment 2: ANOVA $p=0.001$, Clotting time ratios trended lower during treatment: 0 mins (2.0 ± 0.09), 15 mins (1.97 ± 0.11), 30mins (1.89 ± 0.14), $p>0.05$ for all treatment comparison. Start of washout (1.15 ± 0.02) vs. end of washout (1.46 ± 0.06) $p=0.001$ and 30mins vs. both start of washout and end of washout $p=0.001$, $p=0.002$ respectively. Experiment 3: ANOVA $p=0.004$, Clotting time ratios trended lower during treatment: 0 mins

(1.72 ± 0.06), 15 mins (1.5 ± 0.02), 30mins (1.51 ± 0.02), $p > 0.05$ for all treatment comparisons. There was no difference between the start of washout (1.12 ± 0.03) vs. end of washout (1.15 ± 0.01) $p = 0.741$ and 30mins vs. both start of washout and end of washout $p = 0.062$, $p = 0.086$ respectively.

Figure 3. TOP - Histology of biopsies taken from a porcine kidney during experimental treatment with fluorescent labelled (FAM-PTL) tail molecules. Prior to treatment (A); after treatment demonstrating presence of the proteins adherent to the kidney microvasculature (B); and with continued adherence after 6 hours of normothermic reperfusion with blood (C). BOTTOM - Histology of biopsies from a human kidney as part of experiment 5. Prior to treatment with fluorescent labelled (FAM-PTL) tail molecules (D); after treatment with FAM-PTL demonstrating presence of the proteins adherent to the kidney microvasculature (E); and with continued adherence after the further 30 minutes of Washout-2 perfusion (F).

Figure 4. NHRP renal perfusion dynamics in experiments 1-3. Protein Test Group kidneys demonstrated higher renal blood flow rates (PTG1: 30.6 ± 6.8 , PTG2: 27.1 ± 9.4 , PTG3: 25.9 ± 8.6 ml/min/100g) than control kidneys (CG1: 23.1 ± 3.7 , CG2: 20.6 ± 7.6 , TCG3: 17.9 ± 6.2 ml/min/100g; $p = 0.019$, $p = 0.022$, $p = 0.009$ respectively), as well as higher perfusion flow indices PTG1: 0.43 ± 0.12 , PTG2: 0.54 ± 0.22 , PTG3: 0.49 ± 0.16 ml/min/100g/mmHg) vs. control kidneys (CG1: 0.32 ± 0.1 , CG2: 0.44 ± 0.22 , TCG3: 0.34 ± 0.12 ml/min/100g/mmHg; $p = 0.049$, $p = 0.031$, $p = 0.009$).

respectively) during 6 hours of NHRP challenge. Two-tailed paired t-test.
*=significant difference vs. corresponding control group.

Figure 5. Example of postcapture frame analysis (TOP LEFT) of a kidney undergoing OPS assessment (TOP RIGHT) and OPS microcirculatory perfusion dynamics in kidneys at 6 hours of NHRP (charts). Functional capillary density was similar between treated and non treated kidneys (CG2: 19.3 ± 3.1 % vs. PTG2: 20.5 ± 2.0 %, $p=0.402$). TLN treated kidneys recorded vessel diameters were larger (CG2: 7.44 ± 0.81 μm vs. PTG2: 10.77 ± 1.23 μm , $p=0.052$), and RBC velocity (CG2: 272.1 ± 26.3 $\mu\text{m/s}$ vs. PTG2: 403.2 ± 31.2 $\mu\text{m/s}$, $p=0.011$), calculated CBF (CG2: 13.2 ± 3.65 $\mu\text{l/s/mm}^2$ vs. PTG2: 41.9 ± 14.5 $\mu\text{l/s/mm}^2$, $p=0.032$), and the PI (CG2: 2.77 ± 0.98 vs. PTG2: 9.05 ± 2.48 , $p=0.021$) higher compared to control kidneys. The HI calculation revealed similar levels of homogenous perfusion within the areas sampled within each kidney (CG2: 3.21 ± 0.18 AU vs. PTG2: 3.27 ± 0.21 AU, $p=0.791$). Two-tailed paired t-test. *=significant difference vs. control group.

Figure 6. rsMD: Scatter diagram of rsMD readings measured from 1 porcine kidney during HMP and NHRP (Top). NHRP cortical lactate concentrations trended higher in non-treated vs. treated kidneys during (CG2: 3.28 ± 1.04 mM/min vs. PTG2: 1.67 ± 0.28 mM/min, $p=0.072$, bottom left). Photograph of kidney undergoing microdialysis monitoring by insertion of probes into cortex (bottom right).

Table 1. Porcine kidney characteristics.

Table 2. HMP perfusion dynamics, NHRP blood perfusate clotting analysis, and percent weight change in kidneys during HMP and NHRP in Treatment and Control Groups for Experiments 1-3 are illustrated. * Higher levels of D-dimers were observed in control CG1 (14.5 ± 7.9 mg/L) vs. the treated kidneys, PTG1 (9.5 ± 5.1 mg/L, $p=0.051$). Two-tailed paired t-test.

Table 3. Results from Experiment 4: Human kidney graft characteristics, HMP dynamics, weight changes, rsMD levels during NHRP, and NHRP blood perfusate clotting data. There was insufficient sample for PF1.2 testing.

Figure 1

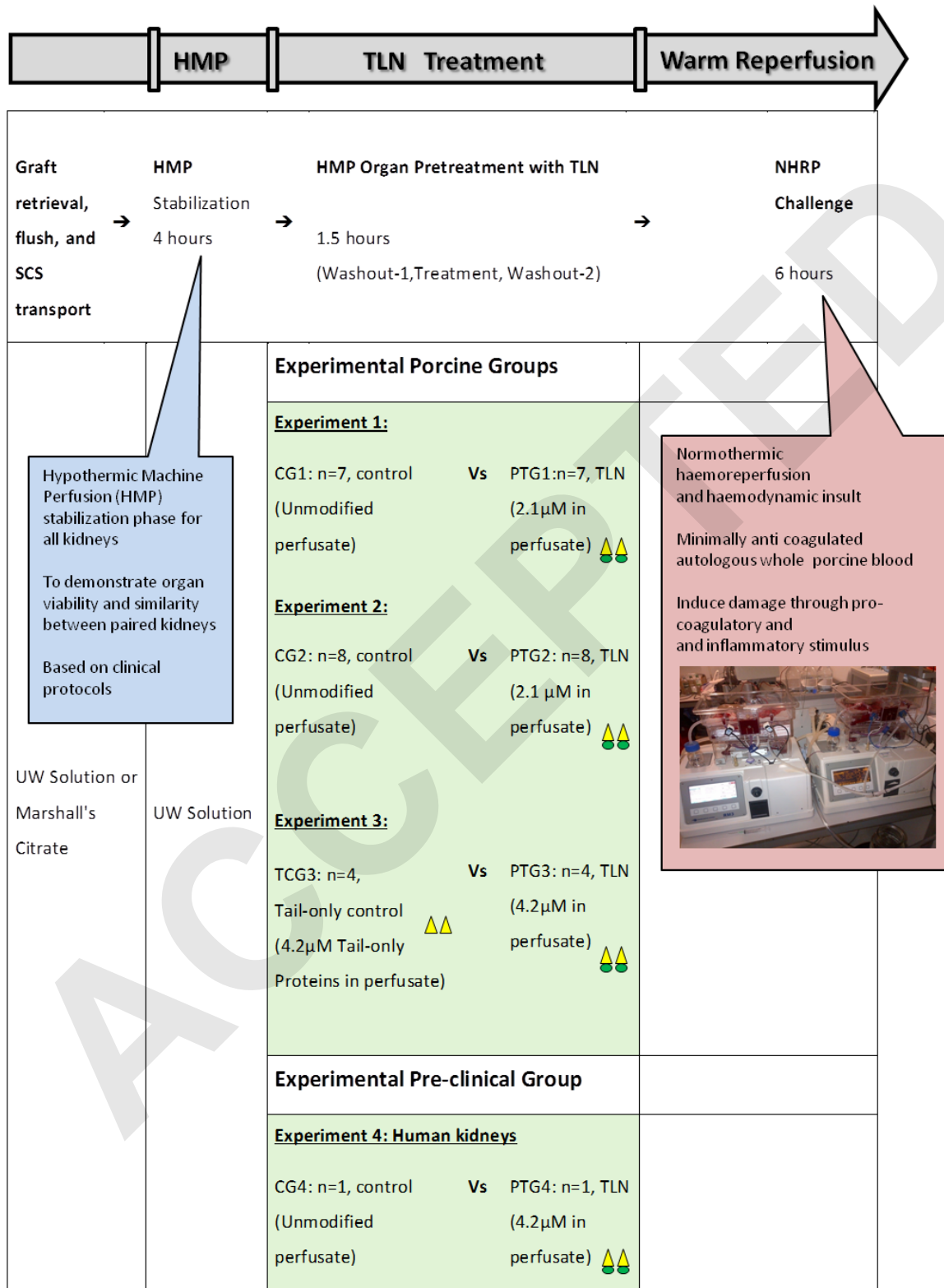


Figure 2

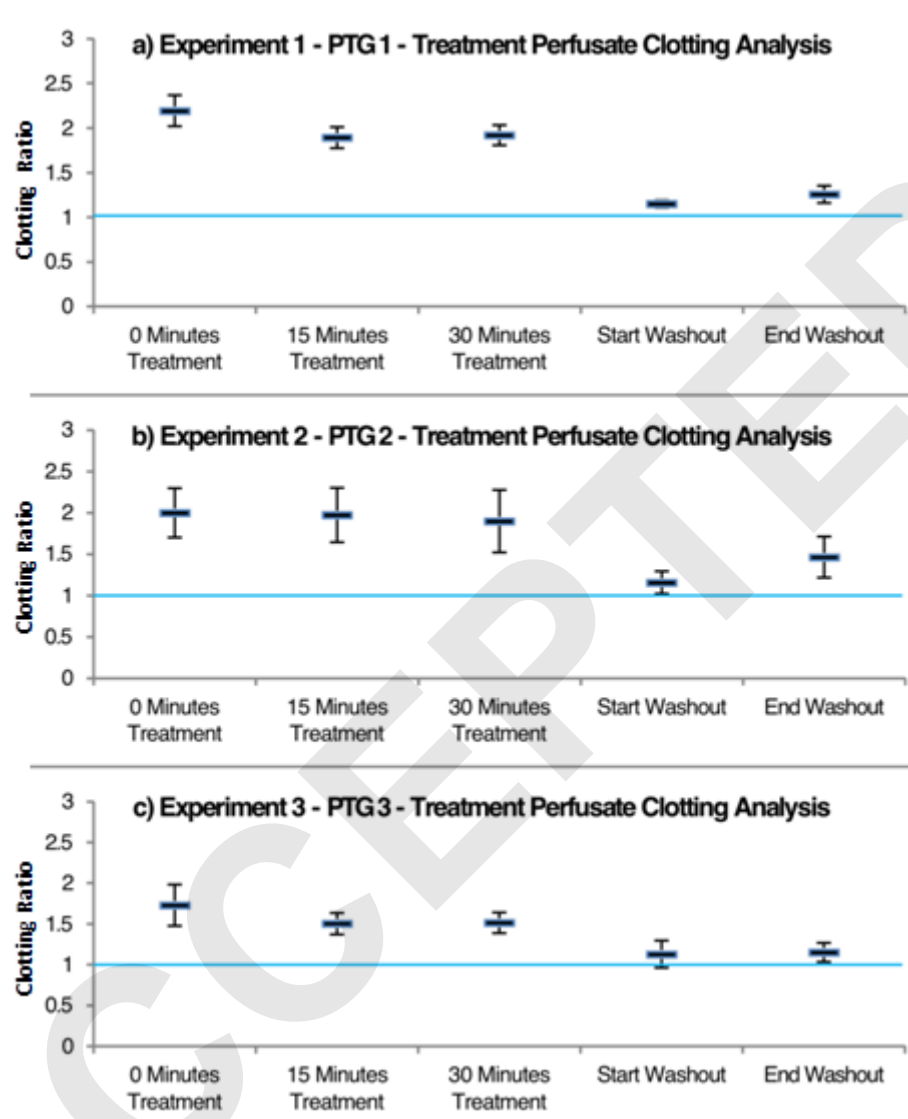


Figure 3

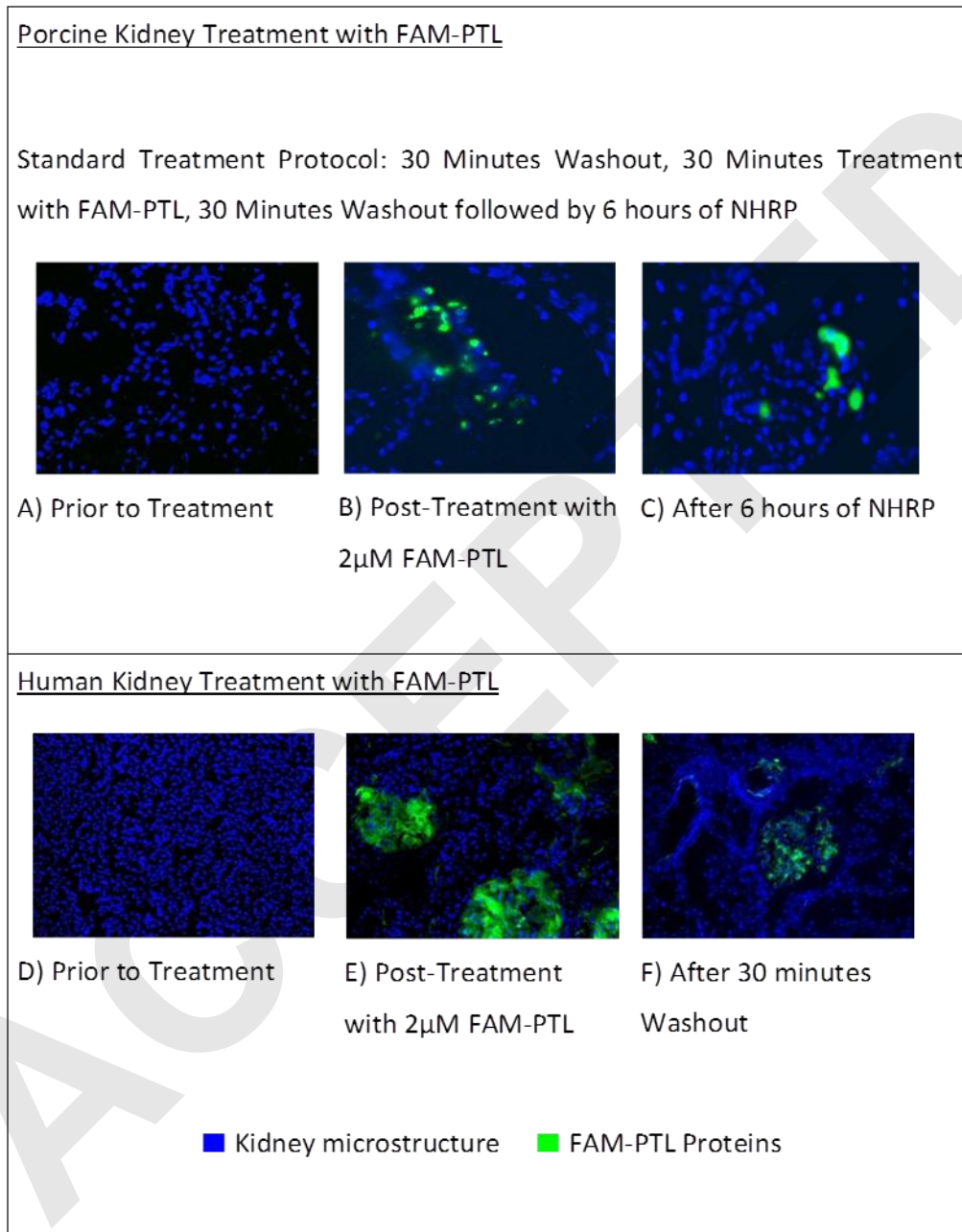


Figure 4

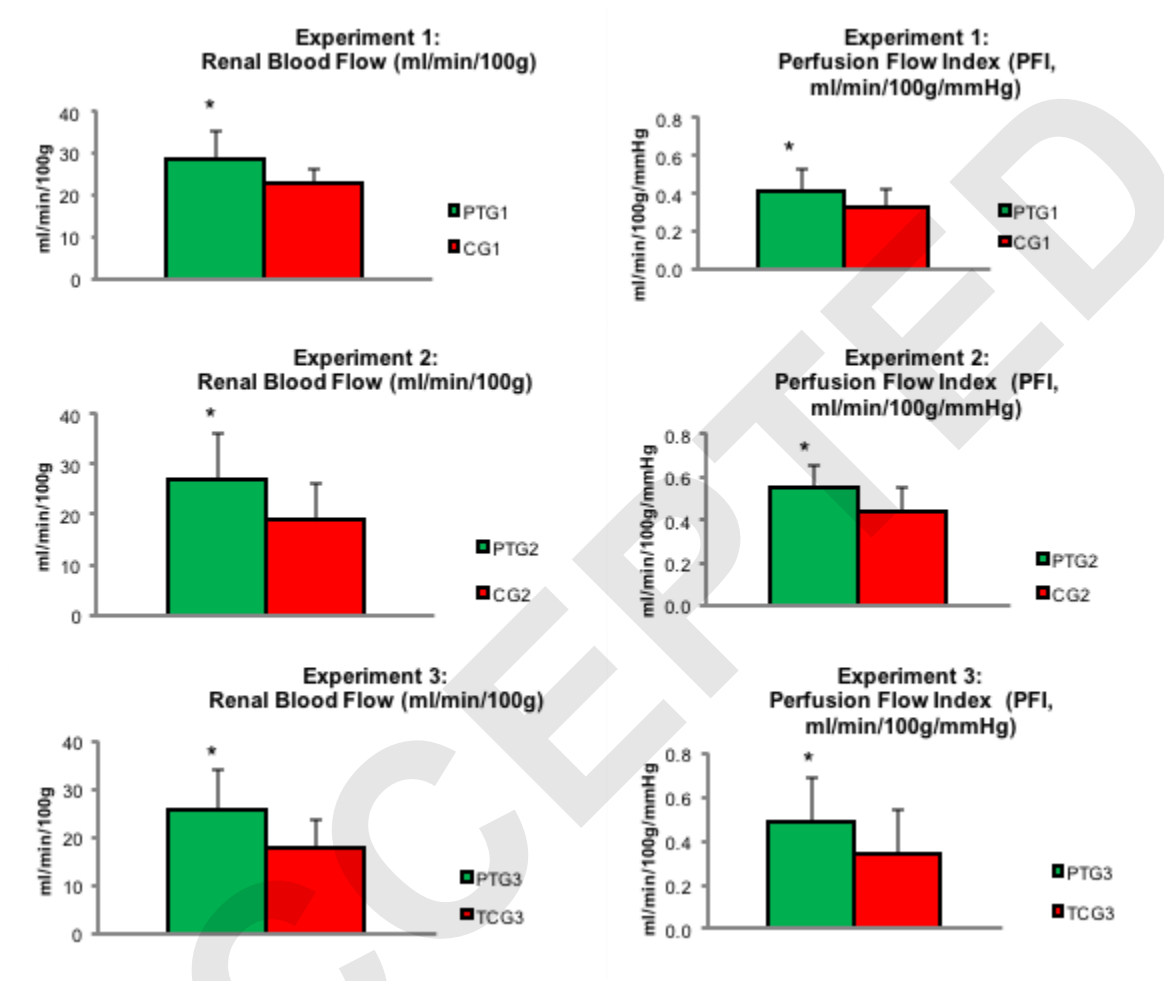


Figure 5

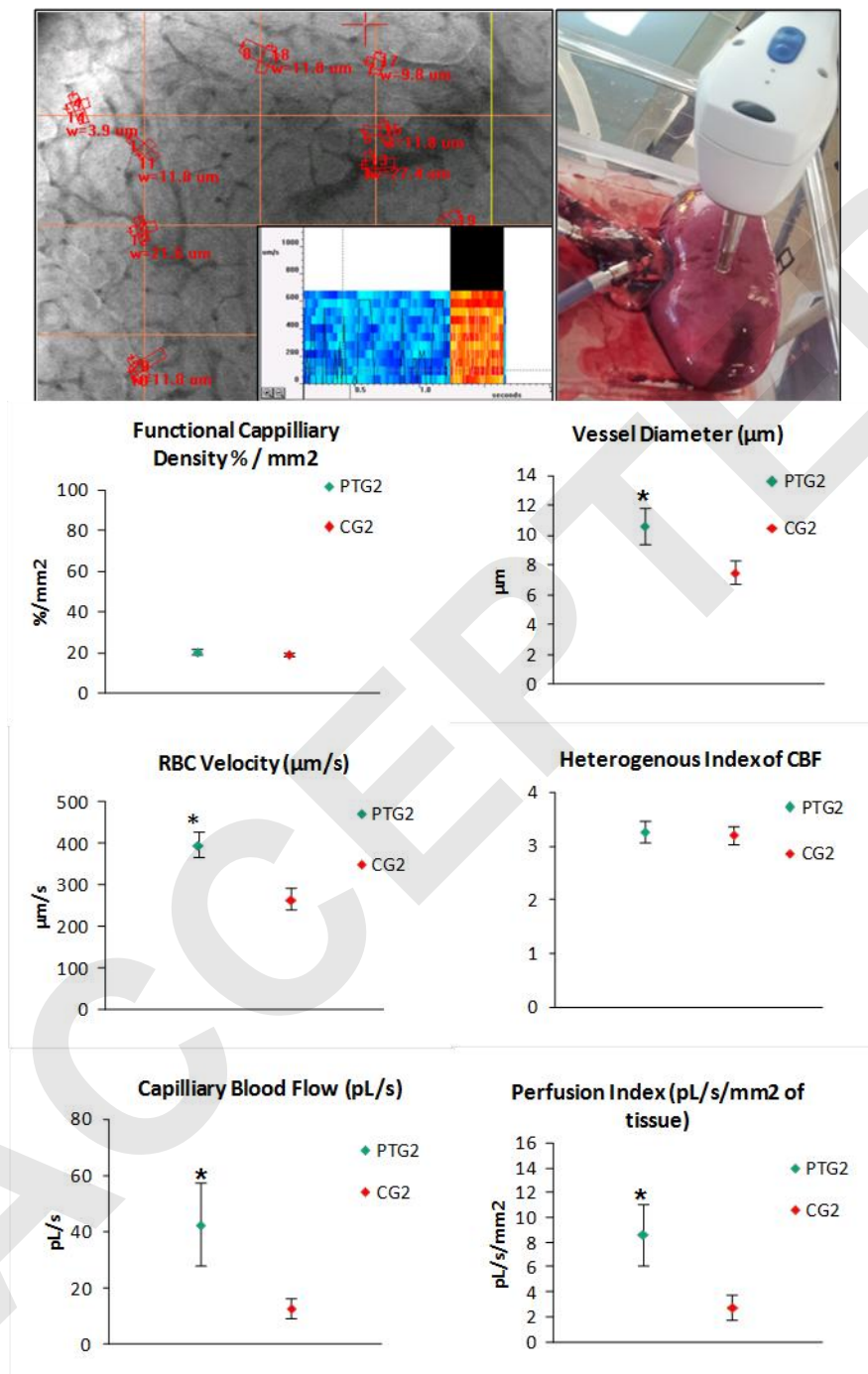
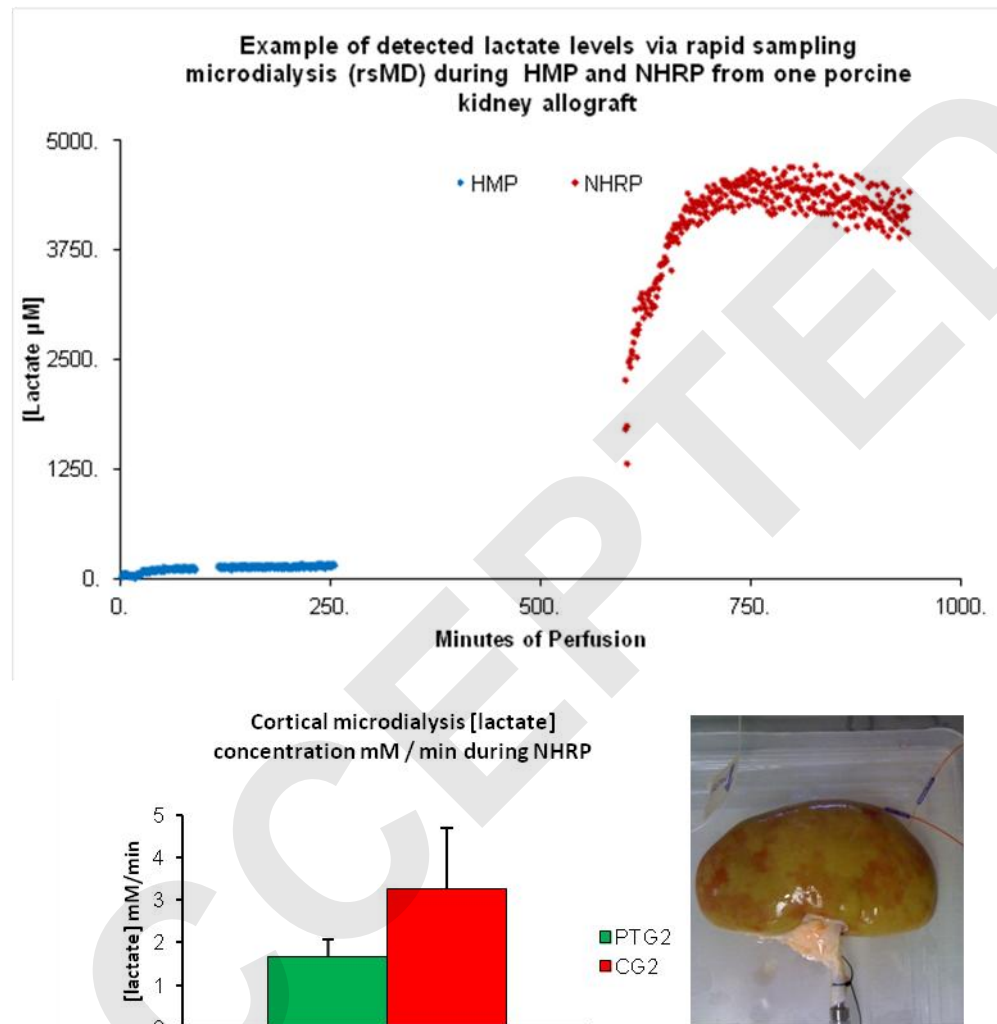


Figure 6



Tables:

Table 1

Kidney Characteristics	Warm Ischaemia Time (minutes) prior to start of SCS		SCS Time (minutes)	Cold Before HMP	Ischaemia HMP Weight Pre-HMP (g)	
Overall for All Kidneys (n=36)	15 (10-20)		298 (219-480)		233 (107-330)	
Experiment 1	CG1	PTG1	CG1	PTG1	CG1	PTG1
	13.6 (2.4)	12.8 (2.6)	345 (80.6)	348 (80.9)	175.1 (229-116)	185.7 (258-107)
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	16 (2)	16 (2)	334 (76)	334 (76)	253 (188-330)	237 (197-315)
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	15.5 (0.6)	15.5 (0.6)	247 (16)	247 (16)	257 (249-295)	251 (240-290)

Table 2

<u>HMP Perfusion Dynamics</u>	Systolic Pressure (mmHg)		Perfusate Flow Rate (ml/min/100g)		Perfusion Flow Index (ml/min/100g/mmHg)	
Experiment 1	CG1	PTG1	CG1	PTG1	CG1	PTG1
	46.2 ± 9.4	43.5 ± 6.3	21.4 ± 6.7	23.4 ± 8.2	0.55 ± 0.22	0.52 ± 0.17
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	38.7 ± 3.8	38.3 ± 3.8	16.2 ± 6.2	16 ± 5.8	0.42 ± 0.08	0.39 ± 0.12
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	36.8 ± 2.7	36.8 ± 1	12.7 ± 3.8	13.6 ± 4.2	0.38 ± 0.08	0.37 ± 0.13
<u>NHRP Perfusate Clotting Analysis</u>	D-dimers (mg/L)		Fibrinogen (µg/L)		Prothrombin Fragment 1+2 (mg/L)	
Experiment 1	CG1 *	PTG1	CG1	PTG1	CG1	PTG1
	14.5 ± 7.9	9.5 ± 5.1	0.385 ± 0.06	0.373 ± 0.21	0.006 ± 0.003	0.007 ± 0.002
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	5.63 ± 2.2	11.2 ± 11.06	1.08 ± 0.19	1.2 ± 0.24	0.005 ± 0.001	0.009 ± 0.002
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	6.18 ± 9.6	6.4 ± 7	0.78 ± 0.07	0.79 ± 0.33	0.002 ± 0.001	0.001 ± 0.002
Percent Weight Change Post HMP (%)			Percent Weight Change Post NHRP (%)			
Experiment 1	CG1	PTG1	CG1	PTG1		
	42.5 ± 12.6	42.8 ± 12.9	10.9 ± 7.8	11.9 ± 9.4		
Experiment 2	CG2	PTG2	CG2	PTG2		
	20.6 ± 7.8	21.8 ± 7.2	9.8 ± 7.9	14.2 ± 14.8		
Experiment 3	TCG3	PTG3	TCG3	PTG3		
	14.8 ± 5.5	14 ± 5.5	19 ± 10	27.8 ± 4.4		

Table 3

Kidney Characteristics	Warm Ischaemia Time (minutes)		Cold Ischaemia Time Before HMP (minutes)		Weight Pre-HMP (g)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	NA	NA	78 hours	78 hours	210	230
HMP Stabilization Perfusion Dynamics	Systolic Pressure (mmHg)		Flow Rate (ml/min/100g)		Perfusion Flow Index (ml/min/100g/mmHg)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	37.8	49.5	18.2	10.2	0.48	0.2
Weight Changes	Percent Weight Change Post HMP (%)		Percent Weight Change Post NHRP (%)			
Experiment 4	CG4	PTG4	CG4	PTG4		
	14.3	15.6	1.2	-0.8		
RSMD [Lactate]	HMP [Lactate] mM/min		NHRP [Lactate] mM/min			
Experiment 4	CG4	PTG4	CG4	PTG4		
	0.17	0.28	6.997	4.442		
Perfusate Analysis	Ddimers (mg/L)		Fibrinogen (µg/L)		Pro-thrombin Fragment 1+2 (mg/L)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	4.74	1.89	3.7	1.2	NA	NA

Methods:

Orthogonal Polarization Spectral (OPS) Imaging

OPS has been validated in animal [1–5] and human models [6,7] and has been used to investigate changes in the microcirculation in transplantation settings [8–11]. Images were recorded for ≥ 2 areas after 6 hours of NHRP in 4 pairs of kidneys. Analysis and quantification of microdynamic parameters was performed offline by frame-to-frame analysis of the recorded images using a computer-assisted image analysis system (CapiScope, KKTechnology, United Kingdom). Analysis included a quantitative assessment of capillary diameters (D), functional capillary density (FCD) and RBC velocity (RBCv). In each sample diagnostic fields (region of interest) each measuring $782 \times 782 \mu\text{m}$ (0.611 mm^2) of interest that were isolated and analysed. FCD was defined as the ratio of area of RBC perfused capillaries per area of observation (mm^2), directly reflecting the state of nutritive tissue perfusion[12]. vRBC ($\mu\text{m/s}$) was measured in the midpoint of ten capillaries that crossed a designated grid within the ROI by means of frame-to-frame analysis (Modification of De Backer's score)[13]. Individual volumetric capillary blood flow (CBF; pl/s) was calculated from RBCv and D metrics according to the formula $\text{vCBF} = [(D/2) \times \text{vRBC}]$. To evaluate changes in overall capillary perfusion, a perfusion index (PI) was calculated from using the FCD and CBF metrics ($\text{PI} = \text{FCD} \times \text{CBF}$, pl/s/mm^2). The degree of homogeneity (heterogeneity index, HI) in CBF was assessed by expressing the standard deviation (SD) relative to the difference between the maximum and minimum of the CBF (obtained from all observations from 1 experiment at the time point).

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